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PRINCIPAL INVESTIGATOR: Brandt Schneider, Ph.D.

CONTRACTING ORGANIZATION: Texas Technical University Health Sciences Center
Lubbock, Texas 79430

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Nearly 180,000 women will develop breast cancer in 1999 and 44,000 will die (1). Cancer is essentially a disease of inappropriate proliferation due to a loss of normal cell cycle controls. We study cell cycle controls in the budding yeast *S. cerevisiae*. We have shown that the formation of active G1-cyclin dependent kinase complexes (Cdks) is rate limiting for cell cycle progression (2). The activity of these complexes is regulated through their association with highly unstable G1 cyclins (3). G1 cyclins are constituitively unstable, and their only essential function is to phosphorylate the B-type Cdk inhibitor, Sic1, and target it for degradation (3-5). Sic1 is one of few known in vivo substrates of yeast G1-Cdks. The amount and phosphorylation state of Sic1 appears to determine the timing and size of cell division. Sic1 mutants lacking several phosphorylation sites are stabilized and completely block cell cycle progression (5,6). Thus, the phosphorylation and subsequent degradation of Sic1 appears to be the critical step in commitment to cell cycle progression. By studying the mechanism of cell cycle control, we can begin to understand how cell cycle defects leads to abnormal proliferation, and how by preventing inappropriate proliferation, we may be able to reduce the incidence of breast cancers.

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Brandt J. Schl August 27, 1995

PI - Signature Date

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Abstract

Nearly 180,000 women will develop breast cancer in 1999 and 44,000 will die [1]. Cancer is essentially a disease of inappropriate proliferation due to a loss of normal cell cycle controls. We study cell cycle controls in the budding yeast *S. cerevisiae*. We have shown that the formation of active G1-cyclin dependent kinase complexes (Cdks) is rate limiting for cell cycle progression [2]. The activity of these complexes is regulated through their association with highly unstable G1 cyclins. G1 cyclins are constitutively unstable, and their only essential function is to phosphorylate the B-type Cdk inhibitor, Sic1, and target it for degradation [3-5]. Sic1 is one of few known in vivo substrates of yeast G1-Cdks. The amount and phosphorylation state of Sic1 appears to determine the timing and size of cell division. Sic1 mutants lacking several phosphorylation sites are stabilized and completely block cell cycle progression [3, 6]. Thus, the phosphorylation and subsequent degradation of Sic1 appears to be the critical step in commitment to cell cycle progression. By studying the mechanism of cell cycle control, we can begin to understand how cell cycle defects leads to abnormal proliferation, and how by preventing inappropriate proliferation, we may be able to reduce the incidence of breast cancers.

Introduction

Cell cycle researchers have long wondered about the molecular nature of the machine that drives cell division. Remarkably, all eukaryotes commit to cell division in the same basic way[7]. Both humans and yeast use Cyclin dependent kinases (Cdks), a catalytic kinase (e.g., Cdc28 in yeast and Cdc2 in humans) and a regulatory cyclin (e.g. Cln3 in yeast and cyclin D1 in humans), to regulate the cell cycle [7, 8]. These proteins are so conserved that the human genes function in yeast [9, 10]. In the budding yeast S. cerevisiae, G1 cells increase in mass until they achieve a critical cell size [11-13]. At critical cell size, an event called Start occurs which commits cells to a full round of division [11-13]. Immediately after Start, cell bud, initiate DNA synthesis and duplicate their spindle pole bodies. Start depends upon G1-Cdk activity (Cln-Cdc28 in yeast) [11-13]. We have found that Clns are rate limiting for Start, and that there exists a critical threshold of Cln that must be exceeded for progress past Start [2]. This threshold changes with growth rate and rapidly growing cells require more Cln for cell division than do slowly growing cells [2]. The work in this proposal has examined the role the B-type Cdk inhibitor, Sic1, has in commitment to cell division. We have shown that Sic1 acts analogously to the RB1 gene, and that only essential function of Cln function in yeast is to phosphorylate Sic1 and target it for degradation [4]. The conservation of function between yeast and human cell cycle genes champions yeast as an excellent model system to study human cell cycle regulation and it's relation to breast cancer. Yeast has been integral to the understanding of how G1 cyclins promote cell division. The purpose of this work has been to address three hypotheses.

Hypotheses Tested

1: Is Sic1 an in vivo substrate of Cln kinase complexes and is this phosphorylation required for its proteolysis?

2: Does Sic1 set the Cln threshold for Start?

3: Are their mutants which can initiate cell budding independent of Cln activity?

The scope of this work is to pursue the yeast as an experimental system for studying how cell cycle controls relate to the causation of breast cancer. Recently, numerous exciting discoveries have closely linked the regulation of the cell cycle to cancer causation D1 [14] [15-22]. The first clearly oncogenic cell cycle gene, the human G1-phase cyclin D1, implicated in the causation of breast cancer, was cloned in *S. cerevisiae* [10]. However, the role and function of cyclins and Cdk phosphorylations remain highly elusive. Two large questions need to be answered: What are the relevant in vivo Cdk substrates for cell cycle progression, and what effect does Cdk phosphorylation have on these substrates? By studying the relationship between an in vivo substrate, Sic1, and the Cln-Cdc28 kinase complexes, we hope to learn more about the cell cycle machinery that initiates cell division.

Body of the Report

A. Technical Objectives:

1. *Characterization of Sic1 turnover.*
    a. *Examine Sic1 abundance and half-life as a function of cell cycle position.*
    b. *Examine the half-life of Sic1 as a function of the presence of Cln.*
    c. *Mutate the potential Cdc28 phosphorylation sites of Sic1 to see if these contribute to turnover.*

2. *Does Sic1 set the Cln threshold?*
    a. *Measure Sic1 levels at different growth rates and correlate to Cln levels.*
    b. *Titrate Sic1 against Clns. Is more cln need for Start if more Sic1 is present?*

3. *Find mutants which uncouple budding from Start*

B. Specific Aims:

1. Show that Sic1 turnover is regulated by Cln kinase complexes and that Sic1 phosphorylation is required for its proteolysis.

The goal for this specific aim is to show that the half-life of Sic1 varies with cell cycle position and that phosphorylation by Cln-Cdc28 kinase complexes regulates Sic1 stability.

Progress Report:

Centrifugal elutriation was used to obtain small-unbudded cells. FACS analysis, budding index, and α-factor execution assays were used to assess cell cycle position. Equivalent amounts of protein extracted from each fraction and Sic1 levels were assessed with a polyclonal

Sic1 antibody and normalized to B-tubulin levels. Preliminary experiments indicate that Sic1 levels are highest in unbudded G1 cells. As cells begin to traverse Start, a slower migrating form of Sic1 becomes apparent, and then Sic1 levels rapidly diminish [4]. Experiments suggest that the slower migrating form of Sic1 is due to phosphorylation presumably by Cln-Cdc28 kinases [4]. However, recent reports indicate that Pho85 kinase complexes can phosphorylate Sic1 [23].

Sic1 abundance has also been measured as a function of Cln abundance. Using both asynchronous cultures and synchronized cultures, we have been able to demonstrate an inverse relationship between Cln abundance and Sic1 abundance. When Cln levels are low, Sic1 levels are high and vice versa (see also section 2). This strongly suggests that Cln levels regulate Sic1 levels. The actual half-life of Sic1 as a function of cell cycle position or in the presence or absence of Cln remains to be measured.

The central hypothesis that is being tested in this section is that phosphorylation of Sic1 by Cln-Cdc28 kinase complexes regulates the stability and thereby abundance and function of Sic1. The best way to test this hypothesis is to identify potential Cln-Cdc28 phosphorylation sites, mutate them singly, or in groups, and examine the effect of Sic1 stability. When this proposal was submitted, we knew that Sic1 was an excellent in vitro substrate for Cln-Cdc28 complexes, and that Cln-Cdc28 complexes appeared to phosphorylate as many as 14 different sites. One of these sites corresponds to the mobility shift discussed above. Since that time, Sic1 phosphorylation sites have been mapped in much greater detail, and it has been demonstrated that substitution of alanine for serine or threonine in two or three of these sites greatly stabilizes Sic1 [6]. In fact, overexpression of these stabilized forms of Sic1 results in a G1-like cell cycle arrest [3, 6]. This is in agreement with our previous observation that the essential function of Cln-Cdc28 kinases is to phosphorylate Sic1 and target it for degradation. Because an extensive analysis of the role of phosphorylation site mutants has already been published [6], we have not pursued this question any further. While it has been shown that substitution of alanine for serine or threonine stabilizes Sic1, it has not been reported if the substitution of an acidic amino acid (e.g., aspartic or glutamic acid) has a role in destabilizing Sic1 as we proposed. Thus experiments are on going to test this hypothesis.

Summary:

We and others have established that the essential role of Cln-Cdc28 kinase complexes is to phosphorylate and target Sic1 for degradation [3-6, 24-27]. It is highly likely that the cumulative effect of these phosphorylations is a general decrease in the half-life of Sic1. While it is known that Sic1 must be phosphorylated to be recognized by the SCF ubiquitin-ligase complex [5, 6, 24-27], it is not yet known at the molecular level how this complex differentiates between phosphorylated and unphosphorylated Sic1. It is worth noting that while all Cln-Cdc28 complexes appear to be able to phosphorylated Sic1 in vivo, cells possessing only the Cln3-Cdc28 kinase complex (e.g. *cln1 cln2 clb5 clb6*) mutants appear to be unable to direct the degradation of Sic1 [2]. This suggests that Sic1 phosphorylations may be qualitatively different.

Future Plans:

At this point, we consider this first specific aim satisfactorily answered, and the bulk of our efforts are being directed at completing specific aims two and three.

2. Find out whether Sic1 sets the Cln threshold for Start.

The goal for this specific aim is to determine whether Sic1 levels vary with the growth rate of the culture and determine whether Sic1 sets the Cln threshold for Start. If Sic1 sets Cln thresholds, then we expect Sic1 abundance to change as a function of growth rate, just as the Cln threshold does. Potential outcomes are: 1) Sic1 levels will, like Cln levels, vary with growth rate (the outcome we expect); or 2) Sic1 levels will be relatively independent of growth rate. If the latter is the case, then at low growth rates the cell must use small amounts of Cln to get rid of large amounts of Sic1. Perhaps this could be accomplished over time. Slow growing cells do indeed have long G1 phases, so this "integration of Cln activity over time" model is possible. We have considered this possibility and have methods to address it.

In part 2b, we have begun to measure what effects an extra copy of Sic1 has on the timing and size of bud and S-phase initiation. Because of Sic1's role in S-phase initiation, we believe that extra copies of Sic1 will delay entry into S-phase. The main uncertainty is whether it will have the same effect on budding. If it does, then Sic1 inhibits Start in general; otherwise, Sic1 is specific for S-phase. This is an important point in light of our "coupling protein" hypothesis (see specific aim 3).

Progress Report:

Rapidly growing cells have and need much more Cln at Start than slowly growing cells [2]. I have shown that Cln thresholds exist, if they are set by Sic1, then I expect Sic1 abundance to change as a function of growth rate, just as the Cln threshold does [2]. In the course of this work, in collaboration with several other laboratories, we were able to demonstrate that Cln proteins are highly unstable molecules in all phases of the cell cycle, that Clb-Cdc28 complexes have little or no role in regulating the stability of Clns, and that Clns abundance is, in fact, the only rate limiting step for cell cycle progression [3].

To determine the effect of growth rate on Sic1 abundance, we measured Sic1 levels in asynchronous and synchronous populations of cells. We were able to demonstrate that Sic1 levels varied dramatically with growth rate. Interestingly, we have found that Sic1 levels are inversely proportional to both the growth rate and the Cln levels of the culture. Rapidly growing asynchronous cells have high Cln levels and low Sic1 levels where as cells growing four times slower have five times as much Sic1 and one-fifth as much Cln. This result is in agreement with our conclusions from Specific aim one. Namely, that Cln levels regulate the stability and thereby the abundance of Sic1. When Cln levels are high, Sic1 levels are low. The situation with synchronized cells is different. By comparing roughly equivalent fractions of G1 cells from rapidly growing and slowly growing cells, we were able to show that rapidly growing cells have more Sic1 than slowly growing cells, but that it appears to be less phosphorylated. This suggests that perhaps a Sic1-specific phosphatase, like Cln-Cdc28 complexes, is more active in rapidly growing cultures. Thus, perhaps not only abundance of Sic1, but also its phosphorylation state is critical in determining the timing of commitment to cell cycle progression.

One simple hypothesis for how Sic1 sets "Cln thresholds" is that increased underphosphorylated Sic1 abundance requires more Cln protein to target it for degradation. We have begun to test this hypothesis in several ways: 1) Do extra wild type copies of Sic1 delay entry into S-phase or delay budding? 2) Do extra wild type copies of Sic1 raise the Cln threshold for Start? 3) Do stabilized Sic1 mutants delay cell cycle entry or raise the Cln threshold for Start? Preliminary experiments have been completed for the first two questions, and experiments are under way to test the third question. In both cases, it appears that extra copies of wild type Sic1 delays entry into S-phase. This again fits with the hypotheses and data listed above and strongly suggests that underphosphorylated Sic1 prevents cell cycle progression. This is very reminiscent of the mechanism whereby the RB1 tumor suppressor gene prevents inappropriate cell cycle progression.

Summary:

We have promising preliminary evidence to indicate that underphosphorylated Sic1 sets the Cln threshold for Start. This places Sic1 at the heart of commitment to cell cycle progression and more firmly establishes its role as acting analogously to the RB1 gene in higher eukaryotes.

Future Plans:

The role of Sic1 phosphorylations and a potential Sic1-specific phosphatase needs to be fleshed out. Do phosphorylation mutants that stabilize Sic1 raise the Cln threshold for Start? Do acidic substitutions destabilize Sic1 and lower the Cln threshold for Start? What is the phosphatase that dephosphorylates Sic1? Experiments are underway that address all of these questions and we expect the answers to be both very interesting and very elucidating in the molecular mechanism of cell cycle progression.

3. Identify genes that couple budding to Start.

Start is a singular event where initiation of budding, S-phase and spindle pole body duplication are coupled and occur simultaneously [11-13]. We know that Sic1 couples S-phase to Start [4]. Perhaps other "coupling proteins" exist. Cln-Cdc28 initiates S-phase by inhibiting and inhibitor. Maybe Cln-Cdc28 also inhibits an inhibitor of budding. To attempt to identify and clone such an inhibitor, we have mutagenized a conditional Cln strain and looked for cells that are able to bud in the absence of Cln. These experiments are just underway, but we have promising mutant candidates, and expect to begin cloning and identifying potential mutants in the next year.

Summary:

The identification of another "coupling protein" that acted as an inhibitor to the initiation of budding would be tremendously useful in our further attempts to understand the molecular mechanisms whereby cell cycle progression is controlled.

Future Plans:

Cell cycle regulated proteolysis appears to be the major mechanism whereby cyclin-Cdk complexes irreversibly drive cell cycle progression [28]. We hope that our genetic screen detailed above and currently underway will identify another "coupling protein" that is intricately involved in the regulation of cell cycle progression. Another cyclin-Cdk complex, Pcl-Pho85 appears to have a role in bud initiation [29], and the Pho81 protein is a known Pho85 kinase inhibitor. Perhaps pho81 is the budding equivalent of Sic1. We are testing the effects of overexpression of PHO81 or PCL-PHO85 kinase complexes, or the deletion of pho81 on the timing of buffing. Alternatively, it could be that Cln-Cdc28 activates a protein which activates budding; this would be harder to identify in this screen. Some mutations might constitutively activate a budding protein. However, if no budding inhibitors are found, we will attempt to clone an activator of budding by identifying genes that on high copy plasmids induce budding in the absence of Clns.

Key research accomplishments:

- *The only essential function of Clns is to phosphorylate and target Sic1 for degradation.*
- *Sic1 abundance is dependent upon cell cycle position, growth rate, and Cln levels.*
- *Sic1 is phosphorylated qualitatively differently by different Cln-Cdk complexes.*
- *Clns are constitutively unstable.*
- *Sic1 abundance and phosphorylation state may determine the timing of Start*

Reportable Outcomes:

- *The work presented here has generated two first author manuscripts.*

Schneider B.L., Patton, E.E., Lanker, S. Mendenhall, M.D., Wittenberg, C., Futcher, B. and Tyers, M. (1998) Yeast G1 cyclins are unstable in G1 phase. *Nature* 395 86-89.

Schneider, B.L., Yang, Q.-H., and Futcher B. (1996) Linkage of replication to Start by the Cdk inhibitor Sic1. *Science* 272, 560-562.

- *In the course of this award, I have been hired as an assistant professor in the department of Cell Biology and Biochemistry at Texas Tech Health Sciences Center*

Conclusions:

The regulation of cell cycle progression is a basic biological problem. However, very little is known about the targets of Cdk phosphorylations, and how commitment to cell division is controlled. We have identified Sic1, a potential analog to the RB tumor suppressor gene, as a key Cdk substrate whose phosphorylation has a central role in regulating proliferation [4]. Cln proteins like cyclin D/E in mammalian cells are highly unstable rate limiting activators of cell cycle progression [3]. Cln and Sic1 levels are intricately related, and it is likely that Sic1 acts to restrain cell cycle. The relationship between cell size, cell growth rate, and Cln activity is an ongoing puzzle, and we will continue to investigate these inter-relationships. A better working model of how Sic1 controls cell cycle progression may go a long way towards understanding how cell cycle control effects the onset of cancer

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